भारतीय मानक
Indian Standard

IS 11069 : 2018

परिष्कृत, विरंजित, हाइड्रोजनीकृत, विंटराइज़्ड एवं निर्गन्धीकृत सोयाबीन का तेल — विशिष्टि

(पहला पुनरीक्षण)

Refined, Bleached, Hydrogenated, Winterized and Deodorized (RBHWD) Soybean Oil — Specification

(First Revision)

ICS 67.200

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Oils and Oilseeds Sectional Committee, FAD 13

FOREWORD

This Indian Standard (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Oils and Oilseeds Sectional Committee had been approved by the Food and Agriculture Divisional Council.

The problem of flavour reversion in soybean oil is a constraint in marketing the product as a direct consumption refined oil. Partial hydrogenation of the refined oil would resolve the problem to a great extent. The major precursor of these off-flavours that differentiate the soybean oil from other oils is the content of linolenic acid. Linolenate's role in flavour reversion is evidenced in the improvement in flavour stability that results when the oil is hydrogenated to remove the linolenate. The lowering of the linolenate content from the value usually found in refined, bleached, hydrogenated, winterized, deodorized soybean oil (RBHWD) results in considerable improvement in stability.

This standard was first published in 1984. The standard was later amended to introduce scheme for labelling environment friendly products to be known as ECO-Mark at the instance of the Ministry of Environment and Forests (MoEF).

This revision has been carried out to harmonize the standard with *Food Safety and Standards Act*, 2006 and Regulations framed thereunder and *Vegetable Oils Grading and Marking Rules*, 1955.

In this revision the following major changes have been made:

a) Limit of aflatoxin has been prescribed for non-ECO marked edible oils also;
b) Aflatoxin is determined using High Performance Liquid Chromatography (HPLC) and Enzyme Linked Immunosorbent Assay (ELISA) instead of Thin Layer Chromatography (TLC) prescribed earlier;
c) Colour of oil (expressed and solvent extracted) is determined using 100 mm cell on the Lovibond scale instead of 5¼ inch cell prescribed earlier and limits have been changed accordingly;
d) Limits of unsaponifiable matter and cloud point have been reduced; and
e) Limits of specific gravity, linolenic acid and trans fatty acids has been incorporated to align with *Vegetable Oils Grading and Marking Rules*, 1955.

In the formulation of this standard, due consideration has been given to *Food Safety and Standards Act*, 2006 and Regulations framed thereunder; *Legal Metrology Act*, 2009 and Rules framed thereunder and the *Essential Commodities Act*, 1955. However, this standard is subject to restrictions imposed under these, wherever applicable.

In reporting the results of a test or analysis made in accordance with this standard, if the final value, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding off numerical values (*revised*)'. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

Indian Standard

REFINED, BLEACHED, HYDROGENATED, WINTERIZED AND DEODORIZED (RBHWD) SOYBEAN OIL — SPECIFICATION

(First Revision)

1 SCOPE

This standard prescribes requirements and methods of sampling and test for refined, bleached, hydrogenated, winterized and deodorized (RBHWD) soybean oil.

2 REFERENCES

The following standards contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below:

IS No.	*Title*
548	Methods of sampling and test for oils and fats
(Part 1) : 1964	Methods of sampling, physical and chemical tests (*revised*)
(Part 2) : 1976	Purity tests (*third revision*)
1070 : 1992	Reagent grade water — Specification (*third revision*)
1448 [P: 21] : 2012 /ISO 2719 : 2002	Method of test for petroleum and its products: [P : 21] Determination of flash point — Pensky Martens close cup method (*third revision*)
1699 : 1995	Methods of sampling and test for food colours (*second revision*)
3470 : 2002	Hexane, food grade — Specification (*first revision*)
10142 : 1999	Polystyrene (crystal and high impact) for its safe use in contact with foodstuffs, pharmaceuticals and drinking water — Specification (*first revision*)
10146 : 1982	Specification for polyethylene for its safe use in contact with foodstuffs, pharmaceuticals and drinking water
10151 : 2014	Specification for polyvinyl chloride (PVC) and its copolymers for its safe use in contact with foodstuffs, pharmaceuticals and drinking water
10325 : 2000	Square tins — 15 kg/litre for ghee, *VANASPATI*, edible oils and bakery shortenings — Specification (*second revision*)
10339 : 2000	Ghee, *VANASPATI*, edible oil tins up to 10 kg/litre capacity — Specification (*second revision*)
10910 : 1984	Specification for polypropylene and its copolymers for its safe use in contact with foodstuffs, pharmaceuticals and drinking water
11434 : 1985	Specification for ionomer resins for its safe use in contact with foodstuffs, pharmaceutical and drinking water
11704 : 1986	Specification for ethylene acrylic acid (EAA) copolymers for their safe use in contact with foodstuffs, pharmaceuticals and drinking water
12247 : 1988	Specification for nylon-6 polymer for its safe use in contact with foodstuffs pharmaceuticals and drinking water
12252 : 1987	Specification for polyalkylene terephthalates (PET & PBT) for their safe use in contact with foodstuffs, pharmaceuticals and drinking water
13576 : 1992	Ethylene menthacrylic acid (EMAA) copolymers and terpolymers for its safe use in contact with foodstuffs, pharmaceuticals and drinking water — Specification
13601 : 1993	Ethylene vinyl acetate (EVA) copolymers for its safe use in contact with foodstuffs, pharmaceuticals and drinking water — Specification
IS/ISO 14718 : 1998	Animal feedings stuffs — Determination of aflatoxin B1 content of mixed feeding stuffs — Method using high performance liquid chromatography

3 DEFINITIONS

For the purpose of this standard, the definitions given in IS 548 (Part 1) and the following shall apply.

3.1 Winterizing — Winterizing is the process of removing certain constituents or impurities from the oil which though soluble at medium and high temperature, crystallize and make the oil turbid or cloudy in cold weather.

3.2 Refined, Bleached, Hydrogenated, Winterized and Deodorized Soybean Oil (RBHWD) — Soybean oil which has been neutralized with alkali, bleached with bleaching earth or activated carbon or both, mildly hydrogenated using the nickel catalyst, reducing the iodine value and then winterized. The solid components that separate out during winterization are filtered through a suitable filter press to separate the solid portion from the liquid portion. The liquid portion so obtained goes to a deodorizer, where it is subjected to a high temperature under high vacuum. Permitted antioxidants and synergists may be added to improve its keeping property.

4 REQUIREMENTS

4.1 Description

The material shall be obtained from good quality soybeans (from the plant *Glycine max* (L) Merrill Syn. *Glycine soja* Sieb. and Zucc., fam. Leguminosae).

4.1.1 Solvent extracted oil shall be obtained from the oleaginous material using solvent hexane conforming to IS 3470.

4.2 The material shall be clear and free from adulterants, sediment, suspended and other foreign matter, separated water and added colouring and flavouring substances.

4.3 Oils shall be free from admixture with other oils, when tested in accordance with **9**, **10, 11, 12, 14, 15** and **16** of IS 548 (Part 2).

4.4 Oils shall not contain aflatoxin, more than 30 µg/kg, when tested by the method prescribed in IS/ISO 14718 or as prescribed in Annex A.

4.5 Metal contaminants and pesticide residues shall not exceed the tolerance limits as prescribed in the *Food Safety and Standards (Contaminants, Toxins and Residues) Regulations*, 2011. Approved international standard test methods from ISO and AOAC shall be followed.

4.6 Only permitted antioxidants and antioxidant synergists not exceeding the quantities specified against each as prescribed under the *Food Safety and Standards (Contaminants, Toxins and Residues) Regulations*, 2011, may be used, if required.

4.7 The material shall also comply with the requirements as given in Table 1.

4.8 Optional Requirement

The material shall also pass the insoluble bromide test as given in Annex E, if agreed to between the supplier and the purchaser.

4.9 Optional Requirements for ECO-Mark

4.9.1 The product shall conform to the requirements of quality as given in **4.1** to **4.7**.

4.9.1.1 The manufacturers shall produce to BIS environmental consent clearance from the concerned State Pollution Control Board as per the norms laid down under the *Water* (*Prevention and Control of Pollution*) *Act*, 1974; *Air* (*Prevention and Control of Pollution*) *Act*, 1981; *Water* (*Prevention and Control of Pollution*) *Cess Act*, 1977 respectively, along with the authorization, if required, under the *Environment* (*Protection*) *Act*, 1986, while applying for ECO BIS Mark.

4.9.1.2 The product shall not contain aflatoxin, more than 5 µg/kg, when tested by the method prescribed in IS/ISO 14718 or as prescribed in Annex A.

Table 1 Requirements for Refined, Bleached, Hydrogenated, Winterized and Deodorized Soybean Oil

(*Clause* 4.7)

Sl No. (1)	Characteristic (2)	Requirement (3)	Method of Test, Ref to (5)
i)	Moisture and insoluble impurities, percent by mass, *Max*	0.1	**5** of IS 548 (Part 1)
ii)	Colour in a 100 mm cell on the Lovibond scale, expressed as ($Y + 5R$), not deeper than	15	**13** of IS 548 (Part 1)
iii)	Refractive index at 40ºC	1.463 0 to 1.467 0	**10** of IS 548 (Part 1)
iv)	Saponification value	189 to 195	**15** of IS 548 (Part 1)
v)	Iodine value (Wijs)	107 to 120	**14** of IS 548 (Part 1)
vi)	Acid value, *Max*	0.5	**7** of IS 548 (Part 1)
vii)	Unsaponifiable matter, percent by mass, *Max*	1.0	**8** of IS 548 (Part 1)
viii)	Flash point (Pensky-Martens), closed, ºC, *Min*	250	IS 1448 [P : 21]
ix)	Hexane, ppm, *Max*	5.00	Annex B
x)	Specific gravity at 30ºC/30ºC	0.917 to 0.921	**11** of IS 548 (Part 1)
xi)	Cloud point, ºC, *Max*	10	Annex C
xii)	Linolenic acid (18 : 3), percent by weight, *Max*	3	Annex D
xiii)	Trans fatty acids, percent by weight, *Max*	5.0	Annex D

4.9.1.3 The product shall not contain any of the toxic metals in excess of the quantities prescribed in Table 2.

Table 2 Limits for Toxic Metals

(*Clause* 4.9.1.3)

Sl No.	Characteristic	Requirement	Method of Test, Ref to
(1)	(2)	(3)	(4)
i)	Lead, mg/kg, *Max*	0.5	**15** of IS 1699
ii)	Arsenic, mg/kg, *Max*	0.5	-do-
iii)	Cadmium, mg/kg, *Max*	1.0	-do-
iv)	Mercury (total) mg/kg, *Max*	0.25	-do-

5 PACKING

5.1 The material shall be supplied in suitable well-closed tin or plastic containers, as agreed to between the purchaser and the supplier. Tin or plastic containers once used, shall not be re-used for packaging of edible oils and fats.

Containers made of plastic materials shall be as per IS 10142 or IS 10146 or IS 10151 or IS 10910 or IS 11434 or IS 11704 or IS 12247 or IS 12252 or IS 13601 or IS 13576.

Containers made of tin shall be as per IS 10325 or IS 10339.

5.1.1 For ECO-Mark, the product shall be packed in such packages which are made from recyclable (that is which can be re-processed to manufacture any useful product) or biodegradable materials.

6 MARKING

6.1 The containers shall be marked in English or Hindi in *Devnagri* script with the following information:

a) Name, trade name, type and grade of the oil;
b) Name and business particulars of the manufacturer;
c) Net quantity of the contents in the container;
d) Batch number, month and year of manufacture;
e) Free from argemone oil in oils of expressed type and refined oil and Grade I oil of solvent extracted type.
f) *Nutritional information* — Nutritional information or nutritional facts per 100 g or 100 ml or per serving of the product shall be given on the label containing the following:
   1) Energy value, in kcal;
   2) Amounts of protein, carbohydrate (specify quantity of sugar) and fat, in gram (g) or ml;
   3) Amount of any other nutrient for which a nutrition or health claim is made:

   Provided that where a claim is made regarding the amount or type of fatty acids or the amount of cholesterol, the amount of saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids, in gram (g), and cholesterol, in milligram (mg), shall be declared, and the amount of trans fatty acid, in gram (g), shall be declared in addition to the other requirement stipulated above.
g) Any other requirement as stipulated under *Food Safety and Standards Act*, 2006 and Regulations framed thereunder and *Legal Metrology Act*, 2009 and rules framed thereunder.

6.2 The container of imported edible oil shall also bear the word, ‘Imported’, as prefix to type and grade of oil.

6.3 The package, label or the advertisement of edible oils and fats shall not use the expressions ‘Super-Refined’, ‘Extra-Refined’, ‘Micro-Refined’, ‘Double-Refined’, ‘Ultra-Refined’, ‘Anti-Cholesterol’, ‘Cholesterol Fighter’, ‘Soothing to Heart’, ‘Cholesterol Friendly’, ‘Saturated Fat Free’ or such other expressions which are an exaggeration of the quality of the product.

6.4 For ECO-Mark the containers shall be marked with the following:

a) List of identified critical ingredients in descending order of quantity, percent by mass, which shall include ‘made from soybean oil’;
b) Brief criteria for which the product has been labelled for ECO-Mark; and
c) Shelf life of the product.

6.5 BIS Certification Marking

The product may also be marked with the Standard Mark.

6.5.1 The use of the Standard Mark is governed by the provisions of the *Bureau of Indian Standards Act*, 1986 and the Rules and Regulations made thereunder. The details of conditions under which the licence for the use of the Standard Mark may be granted to manufacturers or producers may be obtained from the Bureau of Indian Standards.

6.6 ECO-Mark

The product may also be marked with the ECO-Mark, the details of which may be obtained from Bureau of Indian Standards.

7 SAMPLING

Representative samples of the material shall be drawn as given in **3** of IS 548 (Part 1).

ANNEX A

(*Clauses* 4.4 *and* 4.9.1.2)

DETERMINATION OF TOTAL AFLATOXIN BY ELISA

A-1 PRINCIPLE

Antibodies specific to aflatoxins B1, B2 and G1 are immobilized on the filter, and toxin (aflatoxin B1) is labelled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in the test sample. Bound enzyme catalyses oxidation of substrate to form a blue complex. Development of colour indicates that the test sample contains aflatoxin.

A-2 APPARATUS

A-2.1 Antibody Coated Solid Support

A-2.2 Aflatoxin Enzyme Conjugate

A-2.3 High Speed Blender

A-2.4 Variable 100-1 000 µl Micropipettes

A-2.5 Glass Culture Tubes

A-2.6 Filters

A-2.7 Timer

A-2.8 Silicon Carbide Boiling Chips

A-3 REAGENTS

A-3.1 Wash Solution- Phosphate Buffered Saline Solution — Dissolve 0.23 g $NaH_2PO_4.H_2O$, 1.95 g $K_2HPO_4.3H_2O$, 8.70 g NaCl, 0.125 ml Tween 20 and 10 mg thimerosal in 900 ml H_2O, adjust *p*H to 7.2 and dilute to 1 litre.

A-3.2 Buffer — 0.1 percent Bovine serum albumin in phosphate buffer saline solution containing 0.05 percent thimerosal.

A-3.3 Substrate Solution A, tetramethylbenzidine (TMB), (0.4 g/l H_2O), *p*H 8.3.

A-3.4 Substrate Solution B, hydrogen peroxide (0.02 percent H_2O_2 in 0.13 percent aqueous citric acid solution, *p*H 3.0).

A-3.5 Methanol

A-3.6 Hexane

A-3.7 Chloroform

A-3.8 NaH_2PO_4

A-3.9 K_2HPO_4

A-3.10 NaCl

A-3.11 Tween 20

A-3.12 Bovine Serum Albumin

A-4 PROCEDURE

A-4.1 Preparation of Sample

A-4.1.1 Weigh 50 g of sample into blender jar.

A-4.1.2 Mix with 250 ml of 55 percent methanol and 45 percent water (*see* IS 1070).

A-4.1.3 Mix 100 ml hexane and blend for 1 min at high speed.

A-4.1.4 Filter mixture and recover filtrate.

A-4.1.5 Leave for 5 min and remove the lower phase containing methanol water (*see* **A-4.1.2**).

A-4.2 Testing

A-4.2.1 Bring all reagents at room temperature (20 - 23°C).

A-4.2.2 Prepare fresh substrate in small culture tubes by mixing 500 µl substrate solution A with 500 µl substrate solution B for each reaction sites used.

A-4.2.3 Add 100 µl test extract to 200 µl buffer (*see* **A-3.2**).

A-4.2.4 Thoroughly mix the diluted test extract and apply 100 µl diluted test extract to the centre of membrane. Using timer, wait for 1 min.

A-4.2.5 Apply 100 µl (2 drops) enzyme solution to the centre of membrane. Using timer, wait for 1 min.

A-4.2.6 Wash with 1.5 ml (30 drops) wash solution added drop wise.

A-4.2.7 Add the entire content of the substrate solution 1.0 ml from each test tube to each reaction site. Wait 1 min and immediately observe site (centre of cup) for blue colour development (negative) or no colour development (positive).

A-4.3 Interpretation of Results

A-4.3.1 Observe the reaction site (centre of the cup) for a blue colour or no colour development at exactly 1 min after adding the substrate A and B mixture (*see* **A-3.3** and **A-3.4**).

Negative — If the reaction site (centre of the cup) turns light blue or darker, test sample contains total aflatoxin B1, B2 and G1.

Positive — If no blue colour is observed in the reaction site (centre of cup) and reaction site remains completely white (no colour change) for at least 1 min, the test sample contains aflatoxin B1, B2 and G1.

ANNEX B

[*Table* 1, *Sl No.* (ix)]

DETERMINATION OF HEXANE RESIDUES IN OILS AND FATS

B-1 PRINCIPLE

The residual hexane content is the quantity of volatile hydrocarbons remaining in the fats and oils following processing involving the use of solvents. The volatile hydrocarbons are desorbed by heating the sample at 80°C in a closed vessel after addition of an internal standard. After determination of a calibration factor, hydrocarbons in the head space are determination of a calibration factor, hydrocarbons in the head space are determined by gas chromatography using packed or capillary columns. Results are expressed as hexane, in mg/kg (or ppm). The method is applicable to the determination of 'free' volatile hydrocarbons expressed in terms of hexane remaining in animal and vegetable fats and oils after extraction with hydrocarbon based solvents.

B-2 APPARATUS

B-2.1 Gas Chromatograph — Gas chromatograph having,

a) thermostatic column capable of maintaining the desired column temperature within ± 1°C;
b) sample inlet system, separately thermostated which can be maintained at a minimum temperature of 100°C. If a capillary column is used, the inlet system must be capable of a 1/100 split injection. For serial analysis a headspace gas chromatograph with automatic sample injection and tempering bath is satisfactory; and
c) flame ionization detector which can be separately thermostated and maintained at a minimum of 100°C.

B-2.2 Recorder — If a recorder trace is to be used for calculating the composition of the samples analyzed, an electronic recorder of high precision is required or else use electronic integrator (*see* **B-2.3**)

B-2.3 Electronic Integrator, which permits rapid and accurate calculations.

B-2.4 Chromatographic Column, either packed or capillary column with the following minimum requirements:

a) *Packed column* — stainless steel or glass, approximately 2 m long and 3.175 mm internal diameter with acid washed and silanized diatomaceous earth, 150-180 mm particle size (80-100 mesh Chromosorb WAW is suitable), stationery phase — squalene consisting of 10 percent of packing.
b) *Capillary column* — glass or fused silica approx 30 m long and 0.3 mm internal diameter.
c) Stationery phase — Methyl polysiloxane (film thickness 0.2 mm).

B-2.5 Syringe — 1 µl, 10 µ1, 1 000 µl capacity, gas tight.

B-2.6 Septum Vial — 20 ml capacity.

B-2.7 Septa and Aluminium Caps Suitable for Septum Vials Together with Crimping Pliers—The septa must be resistant to oils and solvents (butyl rubber or red rubber is recommended).

B-2.8 Tongs, suitable for holding septum vials.

B-2.9 Heating Bath, with clamps for holding septum vials, thermostatically regulated and capable of maintaining a temperature of 80°C. For continuous operation glycerol is recommended as heating liquid.

B-2.10 Shaking Machine

B-3 REAGENTS

B-3.1 Gases

a) *Carrier* — Helium (preferred for better resolution) or Nitrogen 99.99 percent pure, dried and containing a maximum of 10 mg O_2/kg.

b) *Flame ionization detector* — Hydrogen, minimum purity 99.95 percent, air or oxygen, dry, hydrocarbon free (less than 2 ppm hydrocarbon equivalent to CH_4).

B-3.2 Technical Hexane or Light Petroleum, with a composition similar to that used in industrial extraction or failing these *n*-hexane. For calibration, technical extraction hexane is preferred.

B-3.3 *n*-Heptane Internal standard analytical reagent grade.

B-3.4 Vegetable Oil — Solvent free, freshly refined and deodorized. The oil is to be used for calibration and should be of a similar nature as the sample. It should be free from extraction solvent (less than 0.01 percent).

B-4 SAMPLING AND SAMPLE STORAGE

It is essential that loss of solvent from the sample be prevented. The laboratory sample should be in a completely sealed condition and stored at 4°C. Plastic containers should not be used. Sample analysis should be carried out immediately when the sample container is opened.

B-5 GC OPERATING CONDITIONS

Carrier gas flow depends on the carrier gas and the type of column being used for analysis and should be optimized accordingly. The flow of hydrogen and air or oxygen to the FID should be optimized according to the manufacturer's recommendation. Injector and detector temperatures should be set at about 120°C. The column should be maintained at 40°C.

B-6 PROCEDURE

B-6.1 Determination of the Calibration Factor

Weigh to the nearest 0.01 g, 5 g of solvent free vegetable oil (*see* **B-3.4**) into each of the 7 septum vials. Seal each vial with a septum and cap. By means of a syringe add technical lexane to 6 of the seven vials (in the vial with no added solvent is the blank) according to the following table:

ml/5g	0.5	1	2	4	7	10
mg/100g	67	134	268	536	938	1 340

One vial remains without the addition of solvent.

If *n*-hexane is used for calibration the following table applies:

ml/5g	0.5	1	2	4	7	10
mg/100g	66	132	264	528	924	1 320

Shake the 6 vials containing the solvent in the shaking machine vigorously for 1 h. Using the syringe add 5 µl of internal standard (*see* **B-3.3**) to each of the 7 vials. Successively immerse the vials upto the neck in the heating bath at 80°C at intervals of approximately 15 min. This time interval depends on the duration of the GC analysis which is complete on the elution of the internal standard (*n*-heptane). The samples must be placed in the heating at intervals such that each sample is tempered for exactly 60 min.

Warm the gas tight syringe to 60°C. After tempering at 80°C for exactly 60 min and without removing the vial from the heating bath, use the gas tight syringe and withdraw through the septum 1 000 µ1(1 ml) of the head space above the oil. Inject immediately into the gas chromatograph. For each of the vial containing added solvent a calibration factor *F* may be determined by the following formula.

$$F = \frac{C_S \times A_1}{(A_H - A_B - A_1) \times C_1}$$

where

A_H = total peak area of solvent hydrocarbons including the area of internal standard present in the spiked oil. For identification purposes a typical chromatogram of solvent composition should be obtained. Hydrocarbons which usually make up the technical hexane are 2 methyl pentane, 3 methyl pentane, methyl cyclopentane, cyclohexane, etc. Do not include peaks due to oxidation products which may be present in significant amounts.

A_B = peak area of the solvent hydrocarbons present in the oil to which solvent has not been added (blank) less the peak are of the internal standard.

A_1 = peak area corresponding to the internal standard in the spiked samples.

C_1 = quantity of the internal standard added expressed, in mg/kg, of the oil.

C_s = quantity of technical hexane added to the oil present in the vial expressed, in mg/kg, of the oil.

Express the results to the third decimal place.

Calibration factors of the six standards should be approximately the same. The mean calibration factor should be 0.45, if *n*-heptane is used and 0.57, if cyclohexane is used.

The factor (*F*) so evaluated can be used for determining vial quantities of hexane less than 60 mg/kg. If the value of *F* found for the vial containing 0.5 µl of hexane is significantly below the mean value, this deviation is probably due to difficulty in introducing exactly 0.5 µl and this determination must be either eliminated or repeated. For quantities of hexane between 10 and 20 mg/kg it is better to prepare calibration standards by adding 2 µl of internal standard instead of 0.5 µl.

B-6.2 Sample Analysis

Weigh to the nearest 0.01 g, 5 g of the test sample into a septum vial as quickly as possible and close immediately with a septum and cap. Using a syringe add through the septum exactly 5µl of the internal standard. Shake vigorously by hand for about 1 min and then immerse the vial upto the neck in the heating bath. At 80°C for exactly 60 min. Warm the gas tight syringe to 60°C. After tempering at 80°C for exactly 60 min use the gas tight syringe and take from the vial without removing it from the bath 1 000 µl (1 ml) of the head space above the sample. Immediately inject into the gas chromatograph. Carry out two determinations in rapid succession on each sample.

B-7 CALCULATION

The residual solvent expressed in mg/kg (ppm) is given by the following formula:

$$W = \frac{(A_H - A_1) \times F \times C_1}{A_1}$$

where

A_H = total peak area of solvent hydrocarbons including the area of internal standard. Hydrocarbons which usually make up the technical solvents are 2 methyl pentane, 3 methyl pentane, methyl cyclopentane, cyclohexane, etc. Do not include peaks due to the oxidation products. Some of these products may be present in significant amount.

A_1 = peak area corresponding to internal standard in the sample.

C_1 = quantity of the internal standard added in, mg/kg.

NOTE — For an addition of 5 µl of heptane/5 g of sample C_1 = 680 mg/ kg and C_1 = 750 mg/kg, if cyclohexane is used.

F = calibration factor obtained in procedure

Report as the final result the mean of the results of two determinations.

ANNEX C

[*Table* 1, *SI No.* (xi)]

DETERMINATION OF CLOUD POINT

C-0 GENERAL

Cloud point is that temperature at which, under the conditions of this test, a cloud is induced in the sample caused by the first stage of crystallization.

C-1 APPARATUS

C-1.1 Oil Sample Bottles — 115 ml.

C-1.2 Thermometer

C-1.3 Water bath made up of water or chipped ice and water depending upon the temperature required. The temperature of cloud bath shall neither be less than 2°C nor more than 5 °C below the cloud point. A beaker or sauce pan is a convenient container for this purpose.

C-2 PROCEDURE

C-2.1 The sample must be completely dried before making the test. Heat 60 to 75 g of the sample to 130°C immediately before carrying out the test. Pour about 45 ml of the heated sample into an oil sample bottle.

C-2.2 Begin to cool the bottle and contents in the water bath, stirring enough to keep the temperature uniform. When the temperature has reached a temperature of about 10°C above the cloud point, begin stirring steadily and rapidly in a circular motion so as to prevent supercooling and solidification of fat crystals on the sides or bottom of the bottle.

C-2.3 From this position onwards do not remove the thermometer from the sample, since to do so air bubbles may be introduced which will interfere with the test. The test bottle is maintained in such a position that the upper levels of the sample in the bottle and the water in the bath are about the same.

C-2.4 Remove the bottle from the bath and inspect regularly. The cloud point is that temperature at which that portion of the thermometer is no longer visible, when viewed horizontally through the bottle and the sample.

ANNEX D

[*Table* 1, *Sl No*. (vii) and (viii)]

DETERMINATION OF TRANS FATTY ACIDS

D-1 PRINCIPLE

Fat and fatty acids are extracted from food by hydrolytic methods (acidic hydrolysis for most products, alkaline hydrolysis for dairy products, and combination for cheese). Pyrogallic acid is added to minimize oxidative. Triglyceride, triundecanoin ($C_{11:0}$), is added as internal standard. Fat is extracted into ether, then methylated to fatty acid methylesters (FAMEs) using BF_3 in methanol. FAMEs are quantitatively measured by capillary gas chromatography (GC) against $C_{11:0}$ internal standard. Total fat is calculated as sum of individual fatty acids expressed as triglyceride equivalents. Saturated and monounsaturated fats are calculated as sum of respective fatty acids. Monounsaturated fat includes only *cis* form.

D-2 APPARATUS

D-2.1 Gas Chromatograph (GC), Equipped with hydrogen flame ionization detector, capillary column, split mode injector, oven temperature programming sufficient to implement a hold ramp hold sequence. Operating conditions: temperature (°C): injector, 225; detector, 285; initial temp, 100 (hold 4 min); ramp, 3°C/min; final temperature 240; hold 15 min; carrier gas, helium; flow rate, 0.75 ml/ min; linear velocity, 18 cm/s; split ratio, 200:1.

D-2.2 Capillary Column, separating the FAME pair of adjacent peaks of $C_{18:3}$ and $C_{20:1}$ and the FAME trio of adjacent peaks of $C_{22:1}$, $C_{20:3}$, and $C_{20:4}$ with a resolution of 1.0 or greater. SP2560 100 m × 0.25 mm with 0.20 µm film is suitable.

D-2.3 Mojonnier Flasks

D-2.4 Stoppers — Synthetic rubber or cork.

D-2.5 Mojonnier Centrifuge Basket

D-2.6 Hengar Micro-Boiling Granules

D-2.7 Baskets — Aluminum and plastic.

D-2.8 Shaker Water Bath — Maintaining 70-80°C.

D-2.9 Steam Bath — Supporting common glassware.

D-2.10 Water Bath — With nitrogen stream supply, maintaining 40 ± 5°C.

D-2.11 Wrist Action Shaker — Designed for Mojonnier centrifuge baskets.

D-2.12 Mojonnier Motor Driven Centrifuge — Optional; maintaining 600 × g.

D-2.13 Gravity Convection Oven — Maintaining 100 ± 2°C.

D-2.14 Vortex Mixer

D-2.15 Gas Dispersion Tubes — 25 mm, porosity 'A', extra coarse 175 µm.

D-2.16 Three Dram Vials — About 11 ml.

D-2.17 Phenolic Closed Top Caps — With polyvinyl liner, to fit vials.

D-2.18 Teflon/Silicone Septa — To fit vials.

D-3 REAGENTS

D-3.1 Pyrogallic Acid

D-3.2 Hydrochloric Acid — 12 M and 8.3 M. To make 8.3 M HCL add 250 ml 12 M HCl to 10 ml H_2O. Mix well Store at room temperature (20 - 25°C),

D-3.3 Ammonium Hydroxide — 58 percent (*w/w*).

D-3.4 Diethyl Ether — Purity appropriate for fat extraction.

D-3.5 Petroleum Ether — Anhydrous.

D-3.6 Ethanol — 95 percent (*v/v*).

D-3.7 Toluene — Nanograde.

D-3.8 Chloroform

D-3.9 Sodium Sulfate — Anhydrous.

D-3.10 Boron Trifluoride Reagent — 7 per cent BF_3 (*w/w*) in methanol, made from commercially available 14 percent BF solution. Prepare in the hood.

'**Caution** - BORON TRIFLUORIDE MAY BE FATAL IF INHALED'

D-3.11 Diethyl Ether-Petroleum Ether Mixture — 1 + 1 (*v/v*).

D-3.12 Triglyceride Internal Standard Solution — $C_{11:0}$-triundecanoin; 5.00 mg/ml in $CHCl_3$. Accurately weigh 2.50 g $C_{11:0}$-triundecanoin into 500 ml volumetric flask. Add ca 400 ml $CHCl_3$ and mix until dissolved. Dilute to volume with $CHCl_3$. Invert flask at least 10 additional times. Triglyceride internal standard solution is stable up to 1 month when stored in refrigerator (2 - 8°C)

D-3.13 Fatty Acid Methyl Esters (FAMEs) Standard Solutions

D-3.13.1 *Mixed FAMEs Standard Solution* — Reference mixture containing series of FAMEs, including $C_{18:1}$ *cis* and *trans* (available commercially, or equivalent). To prepare mixed FAMEs standard solution break top of glass vial, open and carefully transfer contents of to 3 dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3 dram glass vial. Dilute to about 3 ml with hexane.

D-3.13.2 *$C_{11:0}$ FAME Standard Solutions* — $C_{11:0}$-Undecanoic methyl ester in hexane. Use only in preparation of individual FAME standard solutions (*see* **D-3.13.3**). To prepare $C_{11:0}$ FAME standard solutions, break top of glass vial open and carefully transfer contents to 50 ml volumetric flask. Wash original vial with hexane to ensure complete transfer and add washings to 50 ml volumetric flask. Dilute to volume with hexane. $C_{11:0}$ FAME standard solution is stable up to 1 week when stored at 0°C.

D-3.13.3 *Individual FAME Standard Solutions* — Standard solutions of each of following FAMEs : $C_{4:0}$-tetranoic methyl ester, $C_{6:0}$-hexanoic methyl ester, $C_{8:0}$-octanoic methyl ester, $C_{10:0}$-decanoic methyl ester, $C_{12:0}$-dodecanoic methyl ester, $C_{13:0}$-tridecanoic methyl ester, $C_{14:0}$-tetradecanoic methyl ester, $C_{14:1}$-9-tetradecenoic methyl ester, $C_{15:0}$-pentadecanoic methyl ester, $C_{15:1}$-10-pentadecenoic methyl ester, $C_{16:0}$-hexadecanoic methyl ester, $C_{16:1}$-9-hexadecenoic methyl ester, $C_{17:0}$-heptadecanoic methyl ester, $C_{17:1}$-10-heptadecenoic methyl ester, $C_{18:0}$-octadecanoic methyl ester, $C_{18:1}$-9-octadecenoic methyl ester, $C_{18:2}$-9, 12-octadecadienoic methyl ester, $C_{18:3}$-9.12,15-octadecatrienoic methyl ester, $C_{20:2}$-11,14-eicosadienoic methyl ester, $C_{20:3}$-11,14,17-eicosatrienoic methyl ester, and $C_{22:0}$-docosanoic methyl ester. Prepare individual FAME standard solutions as follows: Break top of glass vial open and carefully transfer contents to 3 dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3 dram glass vial. Add 1.0 ml $C_{11:0}$ FAME standard solution (*see* **D-3.13.2**) and dilute to total volume of about 3.0 ml with hexane. Individual FAME standard solutions are stable up to 1 week when stored in refrigerator (2-8°C).

D-4 EXTRACTION OF FAT

Accurately weigh ground and homogenized test portion (containing ca 100 - 200 mg fat) into labeled Mojonnier flask. Force material into flask as far as possible. Add ca 100 mg pyrogallic acid (*see* **D-3.1**), and 200 ml triglyceride internal standard solution (*see* **D-3.12**). Add a few boiling granules to flask. Add 2.0 ml ethanol and mix well until entire test portion is in solution. Add 10.0 ml 8.3 M HCl and mix well. Place flask into basket in shaking water bath at 70-80°C set at moderate agitation speed for 40 min. Mix contents of flask on Vortex mixer every 10 min to incorporate particulates adhering to sides of flask into solution. After digestion, remove flask from bath and allow to cool to room temperature (20-25°C). Add enough ethanol to fill bottom reservoir of flak and mix gently.

D-5 METHYLATION

Dissolve extracted fat residue in 2-3 ml chloroform and 2-3 ml diethyl ether. Transfer mixture to 3 dram glass vial and then evaporate to dryness in 40°C water bath under nitrogen stream. Add 2.0 ml 7 per cent BF, reagent (*see* **D-3.10**) and 1.0 ml toluene (*see* **D-3.7**). Seal vial with screwcap top containing teflon/silicone septum. Heat vial in oven for 45 min at 100°C. Gently shake vial ca every 10 min.

NOTE — Evaporation of liquid from vials indicates inadequate seals: if this occurs, discard solution and repeat the entire procedure.

Allow vial to cool to room temperature (20-25°C). Add 5.0 ml H_2O, 1.0 ml, hexane, and ca 1.0 g Na_2SO_4 (**D-3.9**). Cap vial and shake 1 min. Allow layers to separate and then carefully transfer top layer to another vial containing ca 1.0 g Na_2SO_4.

NOTE —Top layer contains FAMEs including FAME of triglyceride internal standard solution.

D-6 GC DETERMINATION

Relative retention time (versus FAME of triglyceride internal standard solution) and response factors of individual FAMEs can be obtained by GC analysis of individual FAME standard solution and mixed FAME standard solution. Inject ca 2 µl each of individual FAME standard solutions and 2µl of mixed FAMEs standard solution. Use mixed FAMEs standard solution to 0ptimize chromatographic response before injecting any test solutions. After all chromatographic conditions have been optimized, inject test solutions from **D-5**.

NOTE — With matrixes of unknown composition, it may be necessary to analyze test portion without addition of internal standard to ensure against interferences. Should interfering peak be found, the area of C_{11} internal standard peak must be corrected before performing calculations. Use 2.0 ml chloroform instead of internal standard solution.

D-7 CALCULATIONS

Calculate retention times for each FAME in individual FAMEs standard solutions (*see* **D-3.13.3)**, by subtracting retention time of $C_{11:0}$ peak from retention time of fatty acid peak. Use these retention times to identify FAMEs in mixed FAMEs standard solution. Use additional FAM E solutions (from the same supplier) when necessary for complete FAME identity verification.

D-7.1 Calculate response factor (R_i) for each fatty acid i as follows:

$$R_i = \frac{Ps_i}{Ps_{C11:0}} \times \frac{W_{C11:0}}{W_i}$$

where

P_s = peak area of individual fatty acid in mixed FAMEs standard solution.

$Ps_{C11:0}$ = peak area of $C_{11:0}$ fatty acid in mixed FAMEs standard solution

$W_{C11:0}$ = weight of internal standard in mixed FAMEs standard solution

W_i = weight of individual FAME in mixed FAMEs standard solution.

NOTE — Peaks of known identity with known relative retention times are listed in Table 3

Table 3 Retention Time of Fatty Acids and Methyl Ester (FAME)

(*Clause* D-7.1)

Fatty Acid (1)	**Retention Time** min **(2)**	**Relative Retention Times (to 11:0 Internal Standard)** (3)
4:0 Butyric	10.49	0.46
6:0 Caproic	12.36	0.54
8:0 Caprylic	15.69	0.68
10:0 Capric	20.39	0.89
11:0 Undecanoic	22.99	1.00
12:0 Lauric	25.58	1.11
13:0 Tridecanoic	28.15	1.22
14:0 Myristic	30.65	1.33
14:1 Myristoleic	32.63	1.42
14:1 *trans*-Myristelaidic	32.01	1.39
15:0 Pentadecenoic	33.04	1.44
15:1 Pentadecenoic	34.98	1.52
16:0 Palmitic	35.41	1.54
16:1 *trans*-Palmitelaidic	36.39	1.58
16:1 Palmitoleic	36.88	1.60
17:0 Margaric	37.54	1.63
17:1 Margaroleic	38.92	1.69
18:0 Stearic	39.78	1.73
18:1 *trans* 6-Petroseienic	40.50	1.76
18:1 *trans*-Elaidic	40.61	1.77
18:1 *trans* 11-Vaccenic	40.72	1.77
18:1 Petroseilenic	40.90	1.78
18:1 Oleic	40.99	1.78
18:1 Vaccenic	41.18	1.79
18:1 Octadecenoic	41.54	1.81
18:2 *trans*-Linoleaidic	41.69	1.81
18:2 *trans* 9-Linolelaidic	42.11	1.83
18:2 *trans*12-Linolelaidic	42.53	1.85
18:2 Linoleic	42.87	1.86
20:0 Arachidic	43.75	1.90
18:3 g-Linolenic	44.25	1.92
20:1 Eicosenic *cis* 5	44.42	1.93
20: 1 Eicosenic *trans* 11	44.45	1.93
20:1 Eicosenic *cis* 8	44.67	1.94
20:1 Eicosenic *cis* 11	44.82	1.95
20:1 Eicosenic *cis* 13	44.99	1.96
18:3 Linolenic	45.02	1.96
18:2 Linoleic-conjugated	45.35	1.97
18:2 Linoleic-conjugated	45.40	1.97
21:0 Heneicosanoic	45.69	1.99
18:2 Linoleic-conjugated	46.18	2.01
18:4 Octadectetraenoic	46.39	2.02
20:2 Eicosadienoic	46.65	2.03
22:0 Behenic	47.46	2.06
20:3 g-Eicosatrienoic	47.94	2.09
22:1 Cetoleic	48.27	2.10
22:1 Erucic	48.50	2.11
20:3 Eicosatrienoic	48.68	2.12
20:4 Arachidonic	48.94	2.13
23:0 Tricosanoic	49.22	2.14
22:2 Docosadienoic	50.17	2.18
24:0 Lignoceric	50.79	2.21
20:5 Eicosapentaenoic	50.96	2.22
24:1 Nervonic	51.92	2.26
22:3 Docosatrienoic	51.98	2.26
22:4 Docosatetraenoioc	52.28	2.27
22:5 Docosapentaenoic	54.75	2.38
22:6 Docosahexaenoic	55.82	2.43

D-7.2 Calculate amount of individual (triglyccrides) (W_{TG}) in test sample as follows:

$$W_{FAMEi} = \frac{Pt_i \times Wt_{C11:0} \times 1.006\,7}{Pt_{C11:0} \times R_i}$$

$$W_{TGi} = W_{FAMEi} \times f_{TGi}$$

where

R = response factor of fatty acid *i*.

Pt_i = peak area of fatty acid *i* in test portion.

$Wt_{C11:0}$ = weight of $C_{11:0}$ internal standard added to test portion, g.

1.006 7 = conversion of internal standard from triglyceride to FAME.

$Pt_{C11:0}$ = peak area of $C_{11:0}$ internal standard in test portion.

f_{TGi} = conversion factor for FAMEs to triglycerides for individual fatty acids (*see* Table 4).

NOTE — If procedure is followed exactly, $Wt_{C11:0}$ should be 0.010 g.

Table 4 Factors (f_{TG}) for Conversion of FAMEs to Triglyceride equivalents

(*Clause* D-6.2)

Fatty Acid (1)	f_{FAi}[1] (2)	**Tri/FAME** (f_{TGi})[2] (3)
4:0 Butyric	0.862 7	0.986 8
6:0 Caproic	0.892 3	0.989 7
8:0 Caprylic	0.911 4	0.991 5
10:0 Capric	0.924 7	0.992 8
11:0 Undecanoic	0.930 0	0.993 3
12:0 Lauric	0.934 6	0.993 7
13:0 Tridecanoic	0.938 6	0.994 1
14:0 Myristic	0.942 1	0.994 5
14:1 Tetradecenenoic	0.941 7	0.994 4
15:0 Pentadecenoic	0.945 3	0.994 8
15:1 Pentadecenoic	0.944 9	0.994 7
16:0 Palmitic	0.948 1	0.995 0
16:1 Hexadecenoic	0.947 7	0.995 0
17:0 Margaric	0.950 7	0.995 3
17:1 Margaroliec	0.950 3	0.995 2
18:0 Stearic	0.953 0	0.995 5
18:1 Octadecenoic	0.952 7	0.995 5
18:2 Octadecdieoic	0.952 4	0.995 4
18:3 Linolenic	0.952 0	0.995 4
18:4 Octadectetraenoic	0.951 7	0.995 4
20:0 Arachidic	0.957 0	0.995 9
20:1 Eicosenic	0.956 8	0.995 9
20:2 Eicosadienoic	0.956 5	0.995 8
20:3 Eicosatrienoic	0.956 2	0.995 8
20:4 Arachidonic	0.956 0	0.995 8
20:5 Eicosapentaenoic	0.955 7	0.995 8
21:0 Heneicosanoic	0.958 8	0.996 1
22:0 Behenic	0.960 4	0.996 2
22:1 Docosaenoic	0.960 2	0.996 2
22:2 Docosadienoic	0.960 0	0.996 2
22:3 Docosatrienoic	0.959 8	0.996 1
22:4 Docosatetraenoic	0.959 5	0.996 1
22:5 Docosapentaenoic	0.959 3	0.996 1
22:6 Docosahexaenoic	0.959 0	0.996 1
23:0 Tricosanoic	0.9620	0.9964
24:0 Lignoceric	0.996 3	0.996 5
24:1 Nervonic	0.963 2	0.996 5

[1] f_{FAi} is the conversion factor for conversion of FAMESs to corresponding fatty acids.

[2] f_{TGi} is the conversion factor for conversion of FAMEs to triglycerides for individual fatty acids.

D-7.3 Calculate weight of each fatty acid (W_i) as follows:

$$W_i = W_{FAMEi} \times f_{FAi}$$

where

f_{FAi} = conversion factors for conversion of FAMEs to their corresponding fatty acids (*see* Table 4).

D-7.4 Calculate percent of *trans* fat in test sample [*w/w*; expressed as sum of only *trans* fatty acids ($C_{14:1}$ Trans Myristelaidic + $C_{16:1}$ Trans Palmitelaidic + $C_{18:1}$ Trans 6 Petroselenic + $C_{18:1}$ Trans Elaidic + $C_{18:1}$ Trans 11 Vaccenic + $C_{18:2}$ Trans Linolelaidic + $C_{18:2}$ Trans 9- Linolelaidic + $C_{18:2}$ Trans 12-Linolelaidic + $C_{18:3}$ Trans Linolenic + $C_{20:1}$ Eicosenic Trans 11)] as follows:

$$\text{Transfat, percent} = \left(\sum\nolimits_{\text{Transfat}} W_i / W_{\text{test portion}}\right) \times 100 \text{ percent}$$

NOTE — Test samples containing hydrogenated fat will yield complicated chromatograms due to large number of isomers formed during hydrogenation process. One general indication of hydrogenation is presence of $C_{18:1}$ *trans* peak(s). *trans* peaks elute prior to *cis,* therefore, include all peaks between $C_{18:1}$ *cis* and $C_{18:2}$ *cis,cis* in calculation of $C_{18:2}$ peak area. Often $C_{18:1}$ *trans* "peak" consists of broad series of peaks [due to positional isomers from hydrogenation]; include all of these in $C_{18:1}$ *trans* peak area.

ANNEX E

(*Clause* 4.8)

TEST FOR INSOLUBLE BROMIDES

E-1 PROCEDURE

E-1.1 Dissolve in a test tube about 6 g of the material in 12 ml of a mixture of equal parts of chloroform and acetic acid.

Add bromine, dropwise, until a slight excess is indicated by the colour, keeping the solution at 20°C. Allow the mixture to stand for at least 15 min and place the test tube in boiling water.

E-1.2 The material shall be taken to have passed the test, if the solution when kept in boiling water, does not become cloudy on account of insoluble bromides.

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